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Reduced Ran expression in Ran$^{+/-}$ fibroblasts increases cytokine-stimulated nuclear abundance of the AP-1 subunits c-Fos and c-Jun

Diep Ngoc Thi Pham [a], Hongyu Luo [a], Jiangping Wu [a,b,*]

[a] Laboratory of Immunology, Centre hospitalier de l'Université de Montréal (CRCHUM) – Hôpital Notre-Dame, Montreal, Quebec, Canada
[b] Nephrology Service and Centre de recherche, Centre hospitalier de l'Université de Montréal (CRCHUM) – Hôpital Notre-Dame, Montreal, Quebec, Canada

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ABSTRACT

Ran (Ras-related nuclear protein), a Ras family GTPase, is involved in multiple cellular functions, including the regulation of DNA replication, cell cycle progression, nuclear structure formation, RNA processing-exportation, and nuclear protein importation. Ran$^{+/-}$ embryonic stem (ES) cells were produced in an attempt to generate Ran null mutant mice. Even after an extremely large number of blastocyst injections, no Ran$^{+/-}$ chimeric mice could be generated. Ran$^{+/-}$ ES cell-derived fibroblasts showed reduced Ran protein expression, and manifested augmented nuclear abundance of AP-1 factors (c-Jun and c-Fos) upon cytokine stimulation. Our experiments demonstrated that intracellular Ran protein levels controlled the nuclear presence of certain transcription factors, such as c-Fos and c-Jun.

1. Introduction

Ran (Ras-related nuclear protein) is an abundant 25-KD protein, about 80% of which is located in the nucleus [1,2]. It is a Ras-related guanine nucleotide-binding protein and a GTPase. Ran is a highly conserved gene in yeasts and mammals [1,3]. Multiple Ran isoforms are located in different genomic loci in mice [4]. Ran and Ran/M2 (an actively transcribed isoform) share 94.6% identity in the nucleotide coding sequence, and 94.0% identity and 98.1% similarity (allowing amino acid substitution) in their peptide sequences [5]. It is likely that multiple isoforms are the result of gene duplication during evolution. Ran is expressed in various tissues whereas Ran/M2 expression is restricted to the testis [5]. Ran protein forms a complex with RCC1 (regulator of chromosomal condensation) [6] and performs critical functions in many cellular events, such as DNA replication [7], cell cycle progression [7], nuclear structure formation [8,9], RNA processing-exportation [10], and nuclear protein importation [11].

Recently, we reported that transgenic (Tg) Ran overexpression compromised T cell functions and reduced c-Jun and c-Fos nuclear import upon activation [12]. To verify, from a different angle, whether Ran expression level preferentially controls the nuclear importation of certain transcription factors, we generated Ran$^{+/-}$ mutant embryonic stem (ES) cells in the present study. Ran$^{+/-}$ fibroblasts derived from Ran$^{-/-}$ ES cells showed decreased Ran expression, and this diminution was associated with increased nuclear abundance of c-Jun and c-Fos upon cytokine stimulation. Our results revealed that Ran expression level is critical in determining the nuclear profusion of certain transcription factors.

2. Materials and methods

2.1. Generation of Ran mutant ES cells

The Ran/TC4 cDNA sequence served as a probe to isolate genomic BAC DNA clone 170M19 from the 129/sv mouse BAC genomic library RPCI-22 (CIHR Genome Research Facility, Hospital for Sick Children, Toronto). The targeting vector was constructed by recombination and routine cloning methods with a 12.5-kb Ran/TC4 genomic fragment from clone 170M19. A 2.8-kb BamHI-EcoRV genomic fragment containing exons 2–6 and a part of exon 7 was replaced by a 5-kb FRT/LoxP-flanked Neo/TK cassette. The final targeting fragment was excised from its cloning vector backbone by Sal I digestion and electroporated into ES cells, followed by G418 selection. The Neo/TK selection cassette was removed by subsequent transient transfection of ES cells with a FLPase expression vector [13,14]. Southern blotting was undertaken with a probe corresponding to the 5′ sequence outside the targeting region, to screen and confirm gene targeting and for successful removal of the Neo/TK selection marker.

* Corresponding author at: Laboratory of Immunology, CRCHUM – Hôpital Notre-Dame, 1560 Sherbrooke St. East, Montreal, Quebec, Canada. Fax: +1 514 412 7596.
E-mail address: jiangping.wu@umontreal.ca (J. Wu).

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2.2. Derivation of fibroblast cells from ES cells

ES cells (clone 4C6-1C11) were dissociated into small clusters with collagenase II (200 U/ml, Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C for 10 min and then cultured in non-adherent bacterial plates (Corning Inc., Corning, NY) in differentiation medium comprising 80% DMEM, 20% FBS, 1% non-essential amino acids, 1 mM L-glutamine, 50 U/ml penicillin/streptomycin, and 50 μM β-mercaptoethanol. After 4 days, the cells were transferred into gelatin-coated plates and cultured for an additional 9 days. They were re-plated in adherent cell culture plates after collagenase II (2 mg/ml) digestion in PBS for 30 min at 37 °C, and cultured in differentiation medium. The cells underwent 2 additional passages when they reached confluence and were then cultured in DMEM containing 10% FBS, 1% non-essential amino acids, 1 mM L-glutamine, and 50 U/ml penicillin/streptomycin. Most of the cells in culture appeared to be fibroblast-like at the end of these 2 passages and maintained this morphology thereafter. They were fed every 2 or 3 days during the whole derivation period, by replacing 50% of spent medium with fresh medium, when they were not being re-plated.

2.3. Immunoblotting

Fibroblasts derived from ES cells were stimulated with tumour necrosis factor-alpha (TNFα, 10 ng/ml, R&D Systems, Minneapolis, MN) and interleukin-1beta (IL-1β, 10 ng/ml, Feldan Bio Inc., Montreal, Quebec, Canada) at 37 °C for various time periods. Cytosolic and nuclear extracts of these cells were prepared with NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Various proteins in these extracts were detected by immunoblotting, as described previously [15]. The following antibodies (Abs) were deployed for immunoblotting: rabbit Abs against histone H3 and Ran (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit Abs against c-Jun, c-Fos and monoclonal antibody (mAb) against β-actin (Cell Signalling, Beverly, MA).

2.4. Electrophoresis mobility shift assay (EMSA)

Nuclear protein (5 μg) was incubated on ice with biotinylated double-stranded oligonucleotides. The sequences of the oligonucleotides used for AP-1 were 5′-CTAGTGATGAGTCAGCCGGATC-3′ and 3′-GATGACTACTCAGTCGGCCTAG-5′ (Integrated DNA Technologies, San Diego, CA). One hundred-fold molar excess of unlabeled double-stranded oligonucleotides was added to some samples to assess binding specificity. The reaction mixture was resolved on 5% non-denaturing polyacrylamide gel with 0.5 × TAE as electrophoresis buffer. The oligonucleotides were transferred to Hybond-N^+ nylon membranes (Amersham, Oakville, Ontario, Canada), and cross-linked at 120 mJ/cm^2 with an UV cross-linker for 12 min. Signals were detected with LightShift Chemiluminescent EMSA kit (Pierce Biotechnology).

3. Results

To test the hypothesis that Ran expression levels differentially control certain protein nuclear importation, we attempted to generate Ran null mutant mice. The gene targeting strategy is illustrated in Fig. 1. $Ran^{+/-}$ 129/sv ES cells were generated successfully (Fig. 2A). We injected the ES cells into an unusually large number of C57BL/6 blastocysts (800 blastocyst injections with 4 different ES cell lines, including 2 with a different targeting construct (data not reported). Yet, not a single chimeric mouse was produced, which was quite surprising since we have successfully and consec-

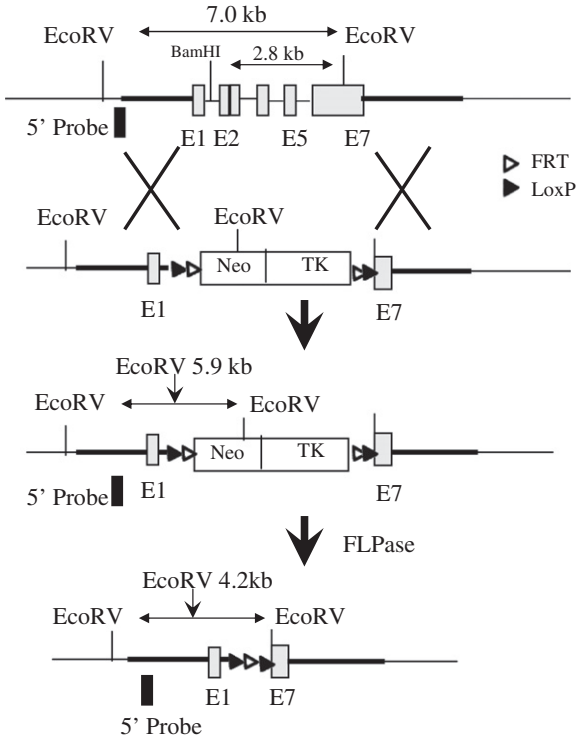

Fig. 1. Targeting strategy to generate $Ran^{+/-}$ ES cells. (A) 2.8-kb BamHI-EcoRV genomic fragment containing exons 2–6 and a part of exon 7 was replaced by a 5-kb FRT/LoxP-flanked Neo/TK cassette. The final targeting fragment was excised from its cloning vector backbone by Sal I and electroporated into ES cells, followed by G418 selection. The Neo/TK selection cassette was removed by subsequent transient transfection of the ES cells with a FLPase expression vector. The black square represents the sequence used as a probe in Southern blotting for genotyping.

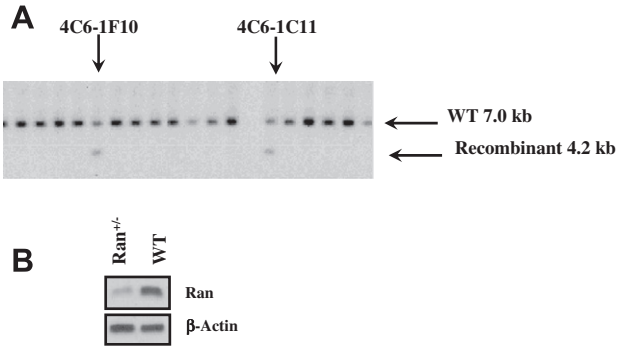

Fig. 2. ES cell genotyping and Ran expression in fibroblasts derived from $Ran^{+/-}$ ES cells. (A) ES cell genotyping by Southern blotting. DNA from targeted ES cells was digested with EcoRV, and analyzed by Southern blotting, with a probe whose sequence location is indicated in Fig. 1. 7.01-kb bands representing the WT region and 4.2-kb bands representing the recombinant region are indicated by arrows. ES cell clones 4C6-1F10 and 4C6-1C11 are $Ran^{+/-}$. (B) Reduced Ran expression in $Ran^{+/-}$ fibroblasts. Fibroblasts were derived from WT and $Ran^{+/-}$ ES cells (clone 4C6-1F10), and their total cellular Ran protein was analyzed by immunoblotting. The blots were stripped and re-probed with anti-β-actin for equal protein loading. The data are representative of 2 independent experiments.

utively generated 30 different gene null mutant mouse lines in recent years, and, on average, we only need to inject about 40 blastocysts to obtain about 5 chimeras. This has raised an intriguing possibility that Ran is so critical in embryonic development that even the deletion of 1 copy of its gene in 129/sv ES cells leads to their failure to compete with wild type (WT) C57BL/6 ES cells in blastocysts.

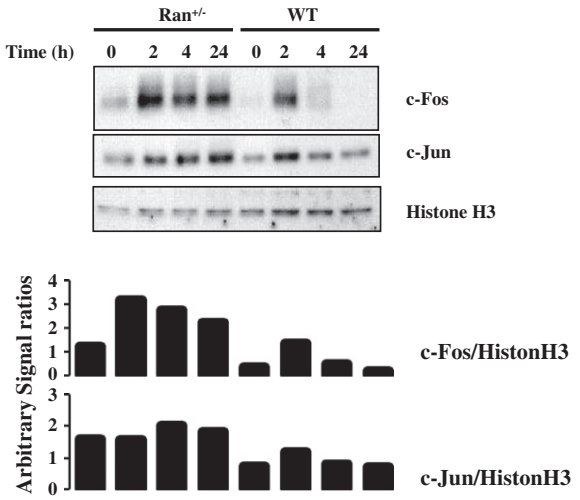

Fig. 3. Increased c-Fos and c-Jun nuclear abundance in Ran$^{+/-}$ fibroblasts compared to that in wild type fibroblasts upon TNFα and IL-1β stimulation according to immunoblotting. Fibroblasts derived from WT and Ran$^{+/-}$ ES cells were stimulated with TNFα (10 ng/ml) and IL-1β 10 ng/ml) for the indicated duration. The nuclear fraction of the cells was analyzed by immunoblotting. Histone H3 levels of the nuclear fraction were tested for equal protein loading. c-Fos and c-Jun levels in the nuclear fraction were studied. The bar graphs in the lower panel show the signals ratios of c-Fos versus histone H3 and c-Jun versus histone H3. The data are representative of at least 3 independent experiments.

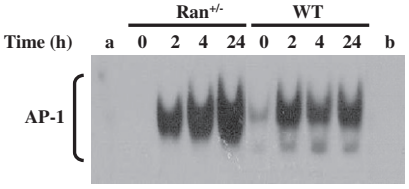

Fig. 4. Increased AP-1 nuclear abundance in Ran$^{+/-}$ fibroblasts upon TNFα plus IL-1β stimulation according to EMSA. The same nuclear proteins extracted from TNFα- plus IL-1β-stimulated WT and Ran$^{+/-}$ fibroblasts, as used in Fig. 3, were investigated by AP-1 EMSA. Lane a is a control with no nuclear protein added. Competition was induced by incubating extracts with a 100-fold molar excess of unlabeled oligonucleotide before addition of the labelled probe (lane b). The data are representative of at least 3 independent experiments.

As an alternative, we derived fibroblasts from WT and Ran$^{+/-}$ ES cells (clone 4C6-1F10), and assessed their transcription factor nuclear import. We confirmed that Ran$^{+/-}$ fibroblasts possessed an obviously lower amount of intracellular Ran, compared to WT fibroblasts (Fig. 2B). When WT fibroblasts were stimulated with TNFα 10 ng/ml) plus IL-1β (10 ng/ml), c-Jun and c-Fos were apparently increased in nuclear fractions (Fig. 3). Interestingly, c-Jun and c-Fos levels were augmented and/or prolonged in the nuclei of Ran$^{+/-}$ fibroblasts compared to WT fibroblasts. The enhanced nuclear presence of c-Jun and c-Fos was confirmed by AP-1 EMSA (Fig. 4). Cytokine stimulation did not change the proliferation rates of either WT or Ran$^{+/-}$ fibroblasts (data now shown).

These results clearly indicate that Ran expression is critical in controlling the nuclear abundance of certain transcription factors: a low Ran level increases the abundance AP-1 factors.

4. Discussion

Ubiquitous Ran expression in the early stages of gestation [12] indicates its critical roles in embryonic development. Since the first report of Ran gene cloning in 1990 [1], profuse literature has been published on its various important functions at the molecular level. However, despite very advanced gene knockout technology in mice in the past decade, the generation of Ran null mutant or Ran$^{+/-}$ heterozygous mice has not been successful so far. Our failed attempt to obtain even chimeric Ran$^{+/-}$ mice suggests that Ran$^{+/-}$ ES cells are not capable of competing with 129/sv cells in blastocysts to establish tissues or organs of Ran$^{+/-}$ ES cell origin. We assessed the proliferation rates of WT and Ran$^{+/-}$ ES cells of similar passages in vitro, but found no significant difference (data not included). Considering the multifaceted functions of Ran, it should not come as a surprise that ES cells with reduced Ran expression are at a disadvantage in various embryonic development processes and fail to evolve further into viable tissues or organs.

Ran is implicated in multiple cellular functions, such as mRNA processing-exportation, protein nuclear importation, DNA replication, cell cycle progression, etc., to name a few [7–11,16]. During cell activation, transcription factors need to increase in abundance in the nucleus to initiate the activation program and then their levels should drop to terminate it. We selected a well-studied transcription factor AP-1 to assess the effect of Ran levels on its nuclear profusion. AP-1 is composed of c-Jun and c-Fos heterodimers, and is essential in multiple signalling pathways. Our previous study showed that the nuclear presence of AP-1 during T-cell activation was significantly reduced in Ran-overexpressing Tg T cells compared to WT T cells [12]. In the present investigation, we demonstrated that AP-1 abundance after cytokine stimulation were augmented in Ran$^{+/-}$ fibroblasts in which Ran expression was reduced because of null mutation in 1 allele. This finding corroborates our previous results on Ran-overexpressing T cells. AP-1 nuclear abundance in T cells and fibroblasts is at the distal end of signalling pathways during T cell and fibroblast activation, and is likely controlled by a similar mechanism, despite different cell types and initial triggering events. Ran is known to be essential in nuclear protein importation. However, our data do not exclude the possibility that the stability or nuclear exportation of AP-1 factors contributes to their increased nuclear abundance. These aspects are worth further investigation.

The heightened nuclear presence of AP-1 members in Ran$^{+/-}$ cells seems contrary to our intuition, as these transcription factors in nuclei are essential in embryonic development. Perhaps the abnormal accumulation of such factors, both in terms of concentration and duration, in the nuclei is detrimental, as are insufficient levels.

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